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ADVANCED DAIRY CHEMISTRY—1: PROTEINS

Edited by

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PREFACE TO THE FIRST EDITION

Because of its commercial and nutritional significance and the ease with which its principal constituents, proteins, lipids and lactose, can be purified free of each other, milk and dairy products have been the subject of chemical investigation for more than a century. Consequently, milk is the best-described in chemical terms, of the principal food groups. Scientific interest in milk is further stimulated by the great diversity of milks—there are about 4000 mammalian species, each of which secretes milk with specific characteristics. The relative ease with which the intact mammary gland can be isolated in an active state from the body makes milk a very attractive subject for biosynthetic studies. More than any other food commodity, milk is a very versatile raw material and a very wide range of food products are produced from the whole or fractionated system.

This text on Proteins is the first volume in an advanced series on selected topics in Dairy Chemistry. Each chapter is extensively referenced and, it is hoped, should prove a useful reference source for senior students, lecturers and research personnel. The selection of topics for 'Proteins' has been influenced by a wish to treat the subject in a comprehensive and balanced fashion. Thus, Chapters 1 and 2 are devoted to an in-depth review of the molecular and colloidal chemistry of the proteins of bovine milk. Although less exhaustively studied than those of bovine milk, considerable knowledge is available on the lactoproteins of a few other species and an inter-species comparison is made in Chapter 3. The biosynthesis of the principal lactoproteins is reviewed in Chapter 4. Chapters 5 to 8 are devoted to alterations in the colloidal state of milk proteins arising from chemical, physical or enzymatic modification during processing or storage, viz. enzymatic coagulation, heat-induced coagulation, age gelation of sterilized milks and chemical and enzymatic changes in cold-stored raw milk. Milk and dairy products provide 20–30% of protein in 'western' diets and are important world-wide in infant nutrition: lactoproteins in particular, are considered in Chapter 9. The increasing significance of 'fabricated' foods has created a demand for 'functional' proteins: Chapters 10 to 12 are devoted to the technology, functional properties and food applications of the caseinates and various whey protein products.

Because of space constraints, it was necessary to exclude coverage of the more traditional protein-rich dairy products: milk powders and cheese. It is hoped to devote sections of a future volume to these products.

I wish to thank sincerely the 13 other authors who have contributed to this text and whose cooperation made my task as editor a pleasure.

P.F. Fox

PREFACE TO THE SECOND EDITION

Considerable progress has been made on various aspects of milk proteins since *Developments in Dairy Chemistry 1—Proteins* was published in 1982. *Advanced Dairy Chemistry* can be regarded as the second edition of *Development in Dairy Chemistry* which has been updated and considerably expanded. Many of the original chapters have been revised and updated, e.g. 'Association of Caseins and Casein Micelle Structure', 'Biosynthesis of Milk Proteins', 'Enzymatic Coagulation of Milk', 'Heat Stability of Milk', 'Age Gelation of Sterilized Milks' and 'Nutritional Aspects of Milk Proteins'. Chapter 1 in *Developments*, i.e. 'Chemistry of Milk Proteins', has been subdivided and extended to 4 chapters: chemistry and physico-chemical properties of the caseins, β-lactoglobulin, α-lactalbumin and immunoglobulins. New chapters have been added, including 'Analytical Methods for Milk Proteins', 'Biologically Active Proteins and Peptides', 'Indigenous Enzymes in Milk', 'Genetic Polymorphism of Milk Proteins', 'Genetic Engineering of Milk Proteins', 'Ethanol Stability of Milk' and 'Significance of Proteins in Milk Powders'. A few subjects have been deleted or abbreviated; the three chapters on functional milk proteins in *Developments* have been abbreviated to one in view of the recently published 4th volume of *Developments in Dairy Chemistry—4—Functional Milk Proteins*.

Like its predecessor, the book is intended for lecturers, senior students and research personnel and each chapter is extensively referenced.

I would like to thank all the authors who contributed to the book and whose cooperation made my task a pleasure.

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CONTENTS

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ANALYTICAL METHODS FOR MILK PROTEINS

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ABBREVIATIONS

ABTS	Ammonium 2,2′-azino-di (3-ethylbenzothiazoline)-6-sulfonate
A & M	Aschaffenburg & Mullen
AOAC	Association of Official Analytical Chemists
AP	Alkaline phosphatase
AUFS	Absorption Units Full Scale
BSA	Bovine serum albumin
CMP	Caseino macropeptide
Cn	Casein
CV	Coefficient of variation
DBC	Dye binding capacity
ELISA	Enzyme-linked immunosorbent assay
FMOC	9-Fluorenyl methyl chloroformate
FPLC™	Fast protein liquid chromatography
FT-IR	Fourier transform infrared
Gu.HCl	Guanidinium chloride
HA	Hydroxyapatite
HI	Hydrophobic interaction
HPLC	High performance liquid chromatography
HTST	High temperature short time
IDF	International Dairy Federation
IEF	Isoelectric focusing
Ig	Immunoglobulin
IR	Infrared

ISO	International Standardization Organization
LF	Lactoferrin (lactotransferrin)
LP	Lactoperoxidase
MESA	Mercaptoethane sulfonic acid
MFGM	Milk fat globule membrane
MIR	Mid-infrared
Mr	Relative molecular weight
NFDM	Non-fat dry milk
NIR	Near-infrared
NPN	Non-protein nitrogen, or non-protein nitrogenous substances
OPA	Orthophtaldialdehyde
PAGE	Polyacrylamide gel electrophoresis
Pg	Plasminogen
PITC	Phenylisothiocyanate
PTC	Phenyl thio-carbamyl
r	Repeatability, or coefficient of correlation
R	Reproducibility, or $1/R$ = reflectance
RP-HPLC	Reversed phase HPLC
RSD_r	Repeatability relative standard deviation
RSD_R	Reproducibility relative standard deviations
s_r	Repeatability standard deviation
s_R	Reproducibility standard deviation
$s_{y,x}$	Accuracy or residual standard deviation
SD or S	Standard deviation
SDS	Sodium dodecylsulfate
SE	Standard error
TCA	Trichloroacetic acid
TNBS	Trinitrobenzene sulfonic acid
UHT	Ultra high temperature
WP	Whey proteins
WPC	Whey protein concentrate
WPI	Whey protein index
α-La	α-Lactalbumin
β-Lg	β-Lactoglobulin
λ	Wavelength

1. Introduction

This review is an up-dated version of the paper recently published by Ribadeau-Dumas and Grappin (1989) on the same subject. It concerns all types of analyses dealing with proteins in milk, dairy and a few non-dairy products. Only the methods that are in current use or those which have been recently published and might become of general use will be considered. For all of them, the reader will be supplied with basic principles, schematic description, references and critical evaluation.

Compared with other food products, milk is a fairly simple fluid. It has been thoroughly studied since the beginning of the 19th century. Its composition and the main characteristics of its various constituents are now well known. In particular, the amino acid sequences of its 7 main protein components have been elucidated. There is today no other food product whose proteins are so well characterized. This makes their analysis straightforward in raw milk. However, as soon as technological treatments have been applied, any quantitative measurement, except nitrogen determination, becomes far more difficult. In particular, protein denaturation, which is not a one-step phenomenon, leads, for a given protein, to products which may differ according to the treatment, often with an ultimate transformation into insoluble aggregates. Furthermore, a number of chemical reactions may occur during the processing of milk, dairy products and non-dairy products which lead to covalent modifications of proteins. In a number of food products, milk proteins have been intentionally fragmented into peptides and amino acids by proteinases and peptidases. Of course it is not possible to know from which protein free amino acids originated. Theoretically, the origin of any peptide with more than 5 amino acids residues, provided it can be isolated, can usually be established if it is derived of any milk protein. However, even the rough characterization of a milk protein hydrolysate is a difficult and long task which can only, for the moment, be performed in few research laboratories. The procedures allowing the determination of milk proteins as a whole and individually in milk and dairy products will be reviewed as well as those used to detect and determine them in non-dairy products. Then, some applications of milk protein analysis in dairy and non-dairy products will be given.

2. Determination of Total Protein in Milk and Dairy Products

As in many other fields, the nature and number of analyses performed to assess the composition of milk and dairy products have changed dramatically during the past 20 years. Fat, which was considered to be the most valuable component in milk since the very beginning of the dairy industry, is now being replaced by proteins which have higher nutrional and economic values. In most countries with a highly developed dairy industry, protein content is now included in milk quality payment schemes and breeding programmes.

Progress in milk protein research, as well as the applications of protein testing to dairy husbandry and quality control in the dairy industry, were for a long time hampered because no rapid and accurate method of analysis was available. A major breakthrough occurred with the introduction of the dye-binding methods in the 1960s, followed by the development of the infrared (IR) techniques which had the advantage of measuring directly all the major milk compounds: fat, protein and lactose.

This chapter will first deal with new developments concerning the determination of nitrogen, mainly by the Kjeldahl method, then the two most important indirect

methods which have been used in dairy laboratories, dye-binding and IR methods, will be described. In central testing laboratories, IR methods have now replaced the dye-binding method. However, because this latter technique is simple, does not require expensive equipment and is still very valuable for quality control in small dairy plants, it will be fully described. Because most of the studies on these techniques have been devoted to milk protein testing, relatively little information is available on their application to dairy products. Extensive reviews on the numerous methods for measurement of the protein content of milk have been published by Bosset *et al.* (1976) and Guillou *et al.* (1987).

2.1. Direct Methods (Nitrogen Determination)

In milk, as well as in other foodstuffs, nitrogen is the element that essentially characterizes proteins. As a consequence, nitrogen determination has always been used as a standard method for the estimation of the protein content of foods.

2.1.1. The Kjeldahl Method

This method is now internationally recognized as the reference method for measuring the protein content of milk products, and is listed as such in the Codex Alimentarius.

2.1.1.1. Principle. In 1883, Kjeldahl discovered that, by heating organic compounds in concentrated sulfuric acid, nitrogen is converted quantitatively into ammonium sulfate and can subsequently be estimated as ammonia by distillation and titration after addition of sodium hydroxide. During digestion, carbon is transformed into CO_2 and hydrogen into H_2O. Detailed information concerning the various reactions involved during digestion can be found in the review of McKenzie and Murphy (1970).

2.1.1.2. Development. Since its discovery, this method has been studied extensively and the procedure revised periodically to improve both the digestion rate and the accuracy. The objective is to convert, as quickly as possible, the totality of the organic nitrogen, even the most refractory compounds, into NH_4^+ without loss of nitrogen by pyrolytic decomposition of ammonia. It is now well established that a high digestion rate and good nitrogen recovery (over 99%) can be achieved only with a suitable ratio of K_2SO_4/H_2SO_4 to reach a high boiling temperature, and with the addition of a catalyst. Several concentrations of K_2SO_4, types of catalyst (selenium, mercury, copper) and oxidizing agent (H_2O_2) have been tested. If, with hard-to-digest compounds, mercury is certainly the most efficient catalyst, it no longer tends to be used because of current environmental concerns. Copper sulfate, which was first proposed by Rowland (1938) for milk analysis, seems to be a good alternative.

Following a study by Rexroad and Cathey (1976), recent studies on foodstuffs (Kane, 1984) and on milk (IDF, 1986) have shown that HgO and $CuSO_4$ give identical results. In the latest version of IDF Standard 20 A/1986 (1986) for the

determination of nitrogen in milk by the macro-Kjeldahl method (5 ml of milk), the following ratios are prescribed: K_2SO_4/H_2SO_4 = 15 g/25 ml; $CuSO_4$, 5 H_2O/H_2SO_4 = 0·05 g/25 ml. Compared to the original $CuSO_4$ concentration recommended by Rowland (1938) (i.e. 0·2 g/5 ml), a 12-fold lower concentration was adopted to prevent the formation of ammoniacal complexes with copper, which may lead to a relative underestimation by about 1% of the nitrogen content.

It is important to bear in mind that a catalyst cannot be exchanged without reconsidering the whole procedure, and especially the K_2SO_4/H_2SO_4 ratio, and the total mineralization time. It is also recommended to consider the total mineralization time given in a Standard Method as a minimum. The heating time should not be reduced if the clearing time (when the digest becomes clear) is short, as for instance with low-fat milk samples. On the other hand, for samples with a high fat or protein content, the amount of H_2SO_4 has to be increased because organic material consumes H_2SO_4 and the total mineralization time should be extended if the clearing time is longer than that given in the Standard Method.

For routine analysis, the classical Kjeldahl flask and gas burner, or electric heater with separate distillation apparatus, are progressively being replaced by block digestion and steam distillation apparatus. These types of equipment give results similar to those obtained with the standard method. However, Barbano *et al.* (1990*a*) found that to achieve good reproducibility, different total mineralization times may be necessary to compensate for differences in line voltage between laboratories.

Preliminary trials (Jeunet, R., 1989, pers. comm.) with a new device, the Maxi-digest (Prolabo, Paris) using microwaves as a means for heating individual flasks gave the same results as the conventional procedure. The Kjel-Fast (CEM Corp., Mattews, NC) which also uses microwaves and mineralizes the sample contained in a quartz vessel in 6 min was tested successfully by Barbano and Clark (1990). Both systems use H_2O_2 to facilitate digestion. The traditional titration using an indicator solution can be carried out by automatic pH titration or specific electrode (Pailler, 1982). The Flow Injection Analysis (FIA) technique which is an automated colorimetric measurement of NH_4^+, has been successfully used by Courroye *et al.* (1989) for the analysis of various nitrogen fractions from cheese. Fully automatic instruments, like the Kjelfoss (Foss-Electric, Hillerod, Denmark), or the Kjel-Auto (MRK Inc., Tokyo, Japan) which combine in a single instrument all the steps of the procedure, i.e. mineralization, distillation and titration, allow the determination of nitrogen with good precision and accuracy at a rate of 20 samples/hour (Grappin & Jeunet, 1976*a*).

2.1.1.3. Analytical performance. When performed correctly, the Kjeldahl method is assumed to give the true nitrogen content of milk. However, many reports of collaborative studies have shown that frequently large discrepancies between laboratories occur. To improve the reproducibility, the new IDF standard requires that two accuracy tests be performed regularly. First, a test based upon the analysis of tryptophan or a similar refractory compound, like phenacetin, to check the mineralization efficiency, with a percentage of N recovery better than 98%.

Lysine-HCl is an alternative, but difficulties have been reported by Kane (1984); nicotinamide, which is also a difficult-to-digest material, does not give an excellent recovery with copper sulfate. Second, a test based on the analysis of an ammonium salt (sulphate or oxalate) is performed to check the distillation and the titration steps, with a percentage of N recovery between 99 and 100%.

To check their procedure, laboratories may take advantage of using reference materials like an $NH_4H_2PO_4$ solution or a milk powder with a certified N content supplied by specialized organizations like the American National Institute of Standards and Technology, or the European Bureau Communautaire de Référence (Brussels).

A collaborative study on the IDF Standard 20 A/1986 (Grappin & Horwitz, 1988), carried out with the participation of 24 reporting laboratories representing 12 countries, found a repeatability relative standard deviation (RSD_r) of 0·5% and a reproducibility relative standard deviation (RSD_R) of 1%; within the United States, Barbano and Clark (1989) found, respectively, RSD_r = 0·31% and RSD_R = 0·44% with 11 participating laboratories.

2.1.2. The Dumas and Related Methods

In these methods, organic and inorganic nitrogen are converted after combustion at high temperature in a furnace into nitrogen gas which is determined by gas chromatography, volumetrically or by thermal conductivity or even chemiluminescence. Automatic apparatus, able to perform fast and reliable analyses (e.g. the Carlo Erba NA 1 500 machine determines nitrogen in 3 min) are commercially available. One machine was tested by Lunder (1974) on casein and whole dried milk and gave reliable data.

Recently, two instrument manufacturers (LECO Corp., St Joseph, USA and Foss-Electric, Hillerod, Denmark) have marketed automated instruments (FP-428 LECO and HERAUS N Analyzer) for the measurement in 3–7 min of nitrogen gas by thermal conductivity via the complete combustion of the food products. A collaborative study on the determination of crude proteins in feeds by the LECO instrument (Sweeney, 1989) has shown good precision (RSD_r = 0·59% and RSD_R = 1·10%). No results are now available concerning the precision of the analysis of liquid products containing relatively low N concentrations.

2.1.3. Conversion Factor—Terminology

To estimate the amount of protein in milk and milk products, it is necessary to convert nitrogen into protein, by multiplying the nitrogen content by a factor, called the Kjeldahl conversion factor. A value of 6·38 for this factor, originally proposed a century ago by Hammarsten and Sebelien on the basis of the nitrogen content (15·67%) of purified acid-precipitated casein, is generally accepted and was confirmed in the latest IDF Standard.

However, this method of determining the protein content raises two important questions. First, the terminology 'protein content' is not fully correct, since the proportion of non-protein nitrogen (NPN) within and between dairy products, varies from 3 to 8% in milk and up to 25–30% in whey. To avoid confusion, the

TABLE 1
Protein Content and Kjeldahl Factor of Milk (Karman & Van Boekel, 1986)

Protein	*Concentration (g/liter)*	*Without carbohydrate*		*With carbohydrate*	
		N%	*Kjeldahl factors*	*N%*	*Kjeldahl factors*
α_{s1}-Cn	10·0	15·77	6·34		
α_{s2}-Cn	2·6	15·83	6·30		
β-Cn	9·3	15·76	6·34		
κ-Cn	3·3	16·26	6·15	15·67	6·38
γ-Cn	0·8	15·87	6·30		
β-Lg	3·2	15·68	6·38		
α-La	1·2	16·29	6·14		
β-SA	0·4	16·46	6·07		
Ig	0·8	16·66	6·00	16·14	6·20
PP,8F,8S	0·5	15·30	6·54		
PP3	0·3	16·97	5·89	15·27	6·55
Lactoferrin	0·1	17·48	5·72	16·29	6·14
Transferrin	0·1	17·00	5·88	16·10	6·21
MFGM	0·4	15·15	6·60	14·13	7·08
Milk	33·0	15·87	6·30	15·76	6·34

term 'crude protein' has to be used to express the nitrogenous matter in milk. Its quantitative expression is represented by the amount of total nitrogen multiplied by 6·38, and is expressed in g per 100 g (or kg or liter) of milk or milk product.

Second, the conversion factor is not constant, but is highly dependent on the amino acid composition of the protein fraction. Recently, using the primary structure of milk proteins, Karman and van Boekel (1986) have shown that for cows' milk, the conversion factor should be 6·34 instead of 6·38, and different factors should be used for casein (6·34), paracasein (6·29), rennet whey proteins (6·45), acid whey proteins (6·30), and NPN (3·60). Recently, these figures were corrected slightly by Van Boekel and Ribadeau-Dumas (1987). For individual protein fractions, the variability of the factor is even greater (Table 1). In their study, they demonstrated that experimental determination of the Kjeldahl factor on (pure) protein fractions leads to substantial discrepancies from the theoretical values obtained from amino acid sequences (Table 2), mainly because it is difficult to obtain pure fractions and to accurately measure the ash content.

Even though the term 'crude protein' and the conversion factor of 6·38 are widely used and accepted for milk and milk products, a more accurate definition and methodology for measuring the 'true' protein content should be promoted. Several authors (Szijarto *et al.*, 1973; Verdi *et al.*, 1987; Robertson and van der Westhuizen, 1990) have pointed out that the NPN content and the NPN/Total N ratio vary extensively according to several biological factors (species, feeding, etc.) and the major proportion of the NPN fraction (urea) has no nutrional nor economic value.